

Effect of Nitrogen Dioxide on Resistance to Respiratory Infection

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INTRODUCTION

In studies of the effect of atmospheric pollutants on health, the basic aspects that must be considered are: the direct damage due to acute and chronic exposure, the role of a pre-existing disease on susceptibility to acute and chronic exposures, and the effects of acute and chronic exposures on resistance to secondary stresses such as respiratory infection.

Air pollutants exert their effect by contact between the pollutant and the body, normally at the surface of skin and exposed membranes. The extent of damage is related to the pollutant's physicochemical properties, its concentration, and the duration of exposure.

For example, among the physicochemical properties, solubility is important. The part of the respiratory system upon which a pollutant may act depends on solubility. A gas of low solubility, such as nitrogen dioxide, penetrates into the lower respiratory tract and exerts its effect in this portion of the respiratory system.

The severity of the tissue response is usually the product of the concentration of the pollutant and the duration of the exposure. Although very low concentrations can sometimes be inhaled for long periods of time without causing any observable effects, inhalation of the same total amount of the gas over a short period of time or as a single breath can result in severe tissue damage and toxic response (14).

The effects of gaseous air pollutants on the membranous surfaces of the respiratory system are of special interest from the standpoint of resistance to respiratory infection. An irritant gas

reaching the epithelium of the trachea or the bronchi can paralyze cilia, alter mucus flow, affect phagocytic activity, and in severe exposures destroy the surface layers of the epithelial lining. These functions constitute the major defense mechanisms and play an important role in respiratory infections.

Nitrogen dioxide is one of the most abundant atmospheric contaminants in many communities. It is emitted in large quantities in the exhausts of automotive engines and is a by-product of natural gas combustion (26). In recent years, it has been increasingly recognized that exposure to oxides of nitrogen (nitrogen dioxide and nitric oxide) can occur in a wide variety of situations. Dangerous accumulations of nitric oxide and nitrogen dioxide can occur, for example, in agricultural silos (17), in enclosed mineshafts after detonation of explosives (3), and in industrial processes requiring the handling of nitric acid (7). A time interval of a few hours after acute exposure usually elapses before symptoms develop (20). After this interval, acute pulmonary edema, cyanosis, severe dyspnea, and bronchopneumonia characteristically develop. When not immediately fatal, the acute episode may be followed by the development of bronchiolitis obliterans, which may cause death during the next few weeks (17) or may lead to persistent abnormalities in airflow.

In the past, the effect of air pollutants on resistance to infection has been studied from two viewpoints, namely, epidemiology and animal experimentation. The discussion in this paper will be limited primarily to the effect of acute and

chronic exposures to the air pollutant nitrogen dioxide on resistance to infection produced by respiratory challenge with airborne *Klebsiella pneumoniae*.

EFFECT OF ACUTE EXPOSURE

The methods used for acute exposure of experimental animals to nitrogen dioxide and for respiratory challenge with aerosols of *K. pneumoniae* have been described in detail in previous publications (21, 22).

Briefly, *K. pneumoniae* type A, strain A-D, was used. It was isolated on Blood Agar Base (Difco) from the heart of an intraperitoneally injected mouse. Stock cultures were prepared on Blood Agar Base in Roux flasks. After 24 hr at 37 C, the growth was harvested in a minimal amount of sterile distilled water and frozen in glass vials containing 2 ml each. For aerosolization, the stock culture was regrown on Blood Agar Base, harvested, and diluted to 10⁸ organisms per milliliter in sterile water.

The aerosol chamber was a 200-liter plastic container which was inserted into a microbiological safety hood. A modified University of Chicago Toxicity Laboratory atomizer was used to produce the aerosol. The liquid culture was fed from a 50-ml syringe, the plunger of which was activated by a revolving threaded rod propelled by a 1-rev/min synchronous electric motor. The atomizer delivered 0.4 ml of culture mixed in 32.5 liters per min of air into the chamber. The chamber air was maintained at 73 ± 2 C and 80 ± 5% relative humidity (RH).

Animals were exposed for 10 min to the bacterial aerosol, in particle size of 1 to 5 μ. After the exposure, aerosol production was stopped, and the animals were air-washed for 15 min.

The source of nitrogen dioxide was a gas cylinder containing 10,000 ppm of nitrogen dioxide in air. The flow of the gas was measured on passage from the cylinder to a mixing chamber where it was further diluted with filtered air. For acute exposures, the nitrogen dioxide-air mixture was introduced into a 3.5-ft³ glass aquarium. For chronic exposure, a walk-in type chamber was used.

Two basic experimental procedures were employed with the use of mice in groups of 10 and hamsters in groups of 6. To determine the effect of pre-exposure to nitrogen dioxide on resistance, experimental animals were exposed to the gas for a 2-hr period before the challenge with the infectious aerosol. To study the effect of nitrogen dioxide on the course of the infection, animals challenged with *K. pneumoniae* were exposed for 2 hr to the gas. The animals were observed for 14

TABLE 1. Mortality of Swiss albino mice exposed for 2 hr to nitrogen dioxide 1 hr before infectious challenge

NO ₂	Mortality (deaths/total)		Change	P
	Infected controls	Exptl group		
ppm			%	
1.5	71/150	86/150	21.1	
2.5	135/400	158/400	16.8	
3.5	44/100	98/100	122.7	<0.05
5	112/250	234/250	108.9	<0.05
10	19/40	39/40	105.3	<0.05
15	13/40	35/40	169.2	<0.05
25	40/100	46/50	130.0	<0.05

days after aerosol challenge, during which time mortality and survival time data were recorded. Autopsies were performed on all animals at the time of death, and randomly selected lung tissues were subjected to histopathological examination. Blood-agar plates were streaked with heart blood to confirm *K. pneumoniae* as the cause of death. Animals surviving the 14-day observation period were sacrificed and examined in the same way.

In all experiments, control groups of animals were exposed either to nitrogen dioxide or to the infectious agent, simultaneously with the experimental animals. Accordingly, results could be compared on the basis of individual test exposures or could be pooled for statistical analysis. The mortality and the survival data were analyzed statistically by the *t* test. Significance is reported at *P* < 0.05. There were no deaths in any of the animals exposed to nitrogen dioxide only. The mortality in the animals challenged with *K. pneumoniae* was only approximately 40%.

Swiss Albino Mice

The effect of a 2-hr exposure to nitrogen dioxide on resistance of Swiss albino mice to infection has been reported in detail in previous publications (8, 9) and will be discussed here only briefly. Table 1 summarizes the data on the effect of nitrogen dioxide in concentrations ranging from 1.5 to 25 ppm. The time interval between the termination of the nitrogen dioxide exposure and the infectious challenge was 1 hr or less. Mice not exposed to the gas but challenged with *K. pneumoniae* aerosol simultaneously with the experimental mice served as controls.

Based on the results shown in Table 1, a threshold value was determined at which the exposure to nitrogen dioxide reduces the resistance of Swiss albino mice to respiratory infection. The threshold is approximately 3 ppm.

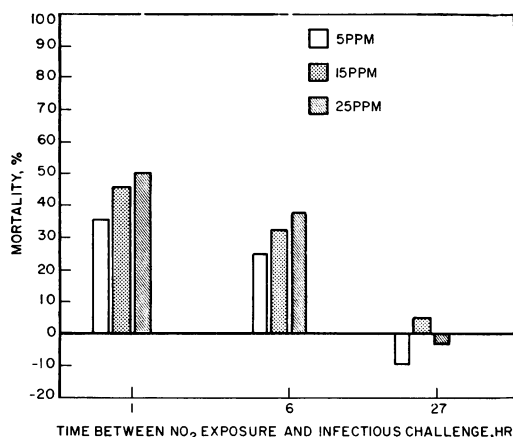


FIG. 1. Mortality rates corrected for control mortality in mice exposed for 2 hr to various concentrations of NO₂ prior to challenge with infectious agent.

The acute exposure appeared to produce an all-or-none response. There was no effect at 2.5 ppm, a complete effect at 3.5 ppm, and a minimal effect at higher concentrations. The mean survival time of the infected controls, calculated on the basis of a maximal 14-day survival, was 11.1 days. The survival time was not affected by exposure to nitrogen dioxide concentrations of up to 2.5 ppm, but it was reduced to 5.5 days at concentrations ranging from 3.5 to 25 ppm.

No deaths occurred in the mice exposed to nitrogen dioxide only, irrespective of the concentrations used. In mice exposed to 5 ppm or more, the lungs were congested to various degrees, and the veins and capillaries of the lungs were dilated. Concentrations of less than 5 ppm produced little, if any, damage.

For the histopathological examinations, the mice were sacrificed within 1 hr after the termination of exposure to nitrogen dioxide. On the few occasions when the sacrifice was delayed for 24 or 48 hr, pathological findings were reduced or absent. Thus, it was of interest to determine whether the effect of acute exposure to nitrogen dioxide on resistance to infection is transitory. To study this parameter, the time interval between the termination of the exposure to nitrogen dioxide and the infectious challenge was extended from 1 hr to 6 and 27 hr. The data in Fig. 1 show that the decrease in resistance was not permanent and disappeared within 27 hr after the termination of the nitrogen dioxide exposure. The persistence of the effect was not influenced by the concentrations of gas used within the 5 to 25 ppm range. It can be assumed, therefore, that nitrogen dioxide produces a temporary damage to

TABLE 2. Mortality of Swiss albino mice exposed for 2 hr to nitrogen dioxide 1 to 24 hr after infectious challenge

NO ₂	Time between challenge and exposure	Mortality		Survival time
		Deaths/total	Per cent	
ppm	hr			days
0	0	27/60	45.0	10.9
2.5	1	20/40	50.0	9.9
2.5	6	33/60	55.0	9.8
2.5	24	32/60	53.3	10.2
0	0	15/30	50.0	10.6
25	1	30/30	100.0 ^a	4.7 ^a
25	6	28/30	93.3 ^a	6.0 ^a
25	24	26/30	86.6 ^a	7.6 ^a

^a Significant at $P < 0.05$.

the defense mechanisms; this damage disappears within 24 hr.

Concentrations of 2.5 or 25 ppm were used to study the effect of a 2-hr exposure to nitrogen dioxide on mortality of previously infected mice. The mortality increased from 50% in the control mice to 100% in the mice exposed to 25 ppm of nitrogen dioxide. At 2.5 ppm, there was no significant difference in mortality between control and exposed mice. Delaying the exposure to nitrogen dioxide for 6 or 24 hr after the infectious challenge did not significantly alter the mortality increase in mice exposed to 25 ppm (Table 2).

Inbred Mice

Increased immunity due to genetically conditioned natural resistance may manifest itself as resistance to invasion by bacteria or as increased ability to produce bacterial antibodies. In studies of acute toxicity of oxides of nitrogen, Gray and co-workers (13) observed appreciable variations in the response of rats obtained from different sources. An exposure difference of 40 ppm was required to produce an LD₅₀ in groups of rats from two different sources. In studies of chronic exposures, Wagner et al. (28) found no effect that could be attributed to the nitrogen dioxide exposures, and therefore strain difference among HLA, C₅₇BL/6, and CAF₁/Jax mice were not observed.

The effect of a 2-hr exposure to 5 ppm of nitrogen dioxide on resistance to respiratory infection was determined in BDF₁, BALB/c, C₅₇BL/c, and LAF₁ mice. Groups of mice from each inbred strain were exposed simultaneously with Swiss albino Webster strain mice to nitrogen dioxide, and either before or after this exposure were challenged with airborne *K. pneumoniae*.

TABLE 3. Mortality of various mouse strains exposed to nitrogen dioxide and infectious challenge

Mouse strain	NO ₂ before challenge			NO ₂ after challenge		
	Mortality (deaths/total)			Mortality (deaths/total)		
	Infected controls	Exptl group	Change	Infected controls	Exptl group	Change
			%			%
SA.....	164/390	262/390	59.6 ^a	247/620	372/620	50.8 ^a
BDF ₁	31/120 ^b	40/120	29.1	66/240 ^b	105/240	58.9 ^a
BALB/c.....	48/100	72/100	50.0 ^a	43/100	62/100	44.2 ^a
C ₅₇ BL/c.....	24/70 ^b	36/70	49.9 ^a	29/130 ^b	34/130	17.4
LAF ₁	40/100	50/100	25.0	52/160	91/160	74.8 ^a

^a Significant ($P < 0.05$) change due to NO₂ exposure.^b Significant ($P < 0.05$) change from Swiss albino mice.

The interval between these two treatments was 1 hr or less.

Table 3 summarizes the results obtained on the basis of a minimum of eight replicate exposures. The data can be considered from two standpoints. One relates to strain differences in resistance to the infection per se; the other, to effects of exposure to nitrogen dioxide on resistance.

BDF₁ and C₅₇BL/c mice were more resistant to the infection than the other two strains, as measured by mortality. The mean survival times of the two more resistant strains were 12.2 and 12.0 days, respectively. Compared with the 10.9 mean survival time of the Swiss albino mice, this increase was statistically significant ($P < 0.05$). The other two inbred strains showed mortalities and mean survival times similar to those of the Swiss albino mice.

Exposure to nitrogen dioxide followed by infectious challenge significantly increased mortality in the Swiss albino, BALB/c, and C₅₇BL/c mice. The increases due to the exposure were 59.6, 50.0, and 49.9%, respectively. The mortality of BDF₁ and LAF₁ mice also increased to 29.1 and 25.0%, respectively, but the differences were not significant. The LAF₁ data, however, must be considered with caution. In this group of experiments, only a small increase in mortality was observed upon exposure to nitrogen dioxide of the Swiss albino mice challenged at the same time as the LAF₁ mice.

Exposure to nitrogen dioxide prior to infectious challenge increased mortality in all five strains. The increase was significant in all but the C₅₇BL/c strain.

The data suggest that mouse strain differences are of importance in resistance to infection produced by *K. pneumoniae*. The damage produced by nitrogen dioxide, on the other hand, is not

TABLE 4. Mortality of hamsters exposed for 2 hr to nitrogen dioxide 1 hr before infectious challenge

NO ₂	Mortality (deaths/total)			P
	Infected controls	Exptl group	Change	
ppm			%	
5	18/96	22/96	21.8	
15	11/126	19/126	73.5	
25	15/72	10/72	-33.2	
35	9/90	40/90	389.0	<0.001
40	6/72	28/72	368.7	<0.005
50	11/138	44/138	298.7	<0.001
65	12/96	61/96	408.0	<0.001

closely related to strain differences. In all instances, mice exposed to nitrogen dioxide either before or after the infectious challenge showed increased mortality. The 5 ppm of nitrogen dioxide did not produce any significant damage to the respiratory system, as determined by histopathological examination of the lungs.

Hamsters

Golden hamsters have a high natural resistance to *K. pneumoniae* infection initiated by the respiratory route. Inhaled respiratory doses as high as 30,000 organisms produced only 12% mortality in our studies; of 690 hamsters challenged with the infectious agent, 82 died. The same challenge dose repeatedly produced 100% mortality in Swiss albino mice used as controls.

A 2-hr exposure to high levels of nitrogen dioxide terminated 1 hr prior to infectious challenge significantly altered the resistance of hamsters. As shown in Table 4, concentrations ranging from 5 to 25 ppm caused some increase in mortality, but it was not significant. Concentra-

tions ranging from 35 to 65 ppm increased mortality significantly; the mortality of the control group was 9.6%, but this increased to 44.7% in the exposed group.

Exposure to nitrogen dioxide apparently is a significant factor in hamsters' resistance to respiratory infection by *K. pneumoniae*. The 10-fold increase in nitrogen dioxide required to produce this effect in hamsters, as compared with mice, cannot be explained at present. It can be related only partially to the differences in body weights and respiratory volumes of these two species. However, the all-or-none response and the absence of a graded dose response are similar in the two species.

Squirrel Monkeys

Increased mortality was observed in preliminary studies with squirrel monkeys exposed for 2 hr to approximately 40 ppm of nitrogen dioxide followed by respiratory challenge. Three groups of monkeys were included in the experiments: one challenged with airborne *K. pneumoniae* only, one exposed to nitrogen dioxide only, and one exposed to nitrogen dioxide and within 1 hr challenged with the infectious agent. Deaths occurred only in the last group; of the five monkeys exposed to both stresses, three died.

EFFECT OF RETENTION OF BACTERIA IN LUNGS

The response of the respiratory system to infectious agents involves the activation of such gross defense mechanisms as cough, alterations in the respiratory functions, phagocytosis, mucus flow, and alterations in ciliary activity.

Under normal conditions, inhaled bacteria are

deposited upon mucus, which, through ciliary action, is constantly moved from the deeper part of the lung toward the larynx. Thus, ciliary movement combined with mucus secretion normally prevents an accumulation of particles in the tracheobronchial tree.

This defense mechanism against invasion by bacteria can be altered. Drying, for example, markedly impairs the mobility and the effectiveness of ciliary actions. Irritant gases, such as ozone, sulfur dioxide, ammonia, and nitrogen dioxide, have been reported to interfere with ciliary movement (2, 6, 15). Thus, one parameter that can be utilized to determine the toxicity or the effect of irritant gases is their action on the ciliated epithelium of the respiratory tract.

The role of phagocytosis as a clearance mechanism of inhaled dust particles is well recognized. Defense against bacterial infection in the lung is similar to defense against dusts. In both cases, alveolar macrophages play a key role in the clearance (16). Green and Kass (11) impaired pulmonary clearance mechanisms in mice by a variety of stresses: hypoxia, cold, corticosteroid injection, and ethyl alcohol intoxication. The inhibition of clearance depended on the type and the extent of the treatment and on the bacterial species being cleared.

To study the effect of nitrogen dioxide on clearance of bacteria by the lower respiratory tract, *K. pneumoniae* was used as the infectious agent. Swiss albino mice and hamsters were exposed for 2 hr to nitrogen dioxide in concentrations ranging from 5 to 50 ppm. Within 1 hr after the exposure, they were challenged with the infectious aerosol. Groups of animals were sacrificed immediately after the infectious challenge. The lungs were removed aseptically from each animal, homogenized in sterile saline, and cultured quantitatively. The initial counts were assumed to be 100% recovery. Control animals as well as animals exposed to the nitrogen dioxide were sacrificed at 1, 3, 5, 6, 7, and 8 hr after the combined treatment. The mean number of bacteria present in the lungs of each group of animals was plotted against the time elapsed after the infectious challenge.

Figure 2 shows the data obtained in mice. Recoveries of *K. pneumoniae* from the lungs of mice exceeding 100% are not shown in the figures. However, they were used in construction of the recovery curves. The mean recovery of bacteria from the lungs of control mice challenged only with the infectious aerosol showed a similar pattern in three replicate tests. The bacterial population was markedly reduced (a range of 65 to 90% was observed) during the first 5 to 6 hr after

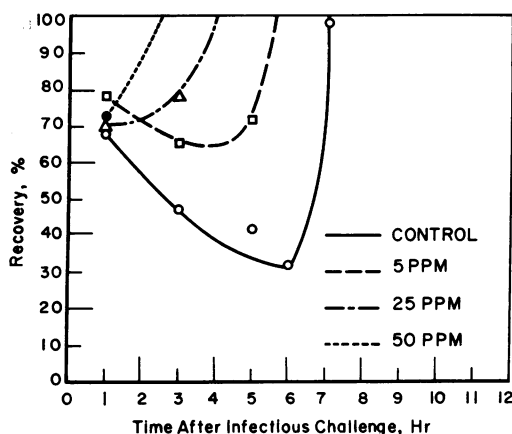


FIG. 2. Recovery of *Klebsiella pneumoniae* from lungs of mice exposed to NO_2 .

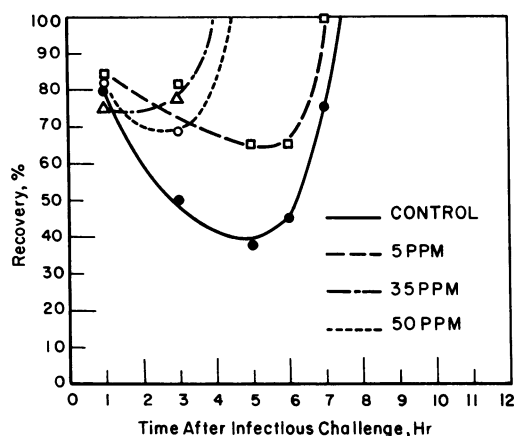


FIG. 3. Recovery of *Klebsiella pneumoniae* from lungs of hamsters exposed to NO₂.

challenge. Thereafter, the population increased and reached the initial concentration after 6 to 8 hr.

In mice exposed to 5 ppm of nitrogen dioxide, the 100% concentration was reached within 5.5 hr. In mice exposed to 25 ppm, the bacterial population decreased during the 1st hr and increased thereafter; the 100% concentration was reached within 3.3 hr. In mice exposed to 50 ppm, the 100% concentration was reached in 2.3 hr. A 4- to 6-log increase in concentration of *K. pneumoniae* occurred in mice 24 hr after the infectious challenge, irrespective of the previous treatment.

Figure 3 shows that similar results were obtained in hamsters. In control hamsters not exposed to nitrogen dioxide, gradual reduction of bacteria occurred during the first 5 hr, and the initial concentration point was reached after 7.3 hr. In hamsters exposed to 5 ppm, this 100% point was observed after 6.4 hr, and in those exposed to 35 ppm, after 2.9 hr.

In the experiments, mice or hamsters exposed to nitrogen dioxide 1 hr before infection were challenged with the infectious aerosol simultaneously with control animals not exposed to the gas. Both groups of animals were thus exposed to the same quantity of *K. pneumoniae*. However, as shown in Table 5, the initial recoveries of *K. pneumoniae* from lungs varied widely. In all instances, fewer organisms were recovered from the animals exposed to nitrogen dioxide. In hamsters, the decrease in organisms appears to be related to the concentration of nitrogen dioxide. At 5 ppm, the recovery was 75% of that in the controls; at 35 ppm, 58%; and at 50 ppm, 44%. However, experiments were not conducted to determine the statistical significance of this relation. In mice, the recovery was approximately

71% of that in controls, irrespective of the nitrogen dioxide concentration.

While the increased mortality in animals exposed to nitrogen dioxide can in part be explained by damage to the ciliary activity and the phagocytic activity, the lower recovery of inhaled bacteria from the lungs of animals exposed to nitrogen dioxide cannot be ascribed to this type of damage. Also, because of the absence of appreciable pulmonary edema in animals exposed to nitrogen dioxide, a dilution effect can be discounted. Three theoretical explanations are possible. One is that the nitrogen dioxide remaining in the lungs inactivates the bacteria in situ. This supposition is questionable, because larger amounts of nitrogen dioxide are usually required to produce any effects on bacteria. Another explanation is that a protective film forms in the respiratory system as a result of inhalation of the gas; this film would make the recovery of bacteria from the lung tissue more difficult. The third possibility is that the respiratory functions are modified by inhalation of nitrogen dioxide.

Our studies in squirrel monkeys showed that exposure to nitrogen dioxide increased the respiratory rate and decreased the tidal volume. Although the animal was breathing more frequently, the breathing was shallow. Thus, it is possible that the bacteria do not penetrate into the alveoli in the same quantities as in normal animals.

The tidal volume of a monkey exposed for 2 hr to nitrogen dioxide is shown in Fig. 4. The monkey was placed in a restraining chair, a mask was fastened to its face, and it was exposed to filtered air for 30 min. Without any interruption, 35 ppm of nitrogen dioxide was introduced into the air, and the respiratory functions were measured with a spirometer and a dual-channel recorder. After the exposure, the tidal volume was approximately 72% of the initial value.

EFFECT ON LACTIC DEHYDROGENASE (LDH) ISOENZYMES

The LDH enzyme system plays a principal role in the glycolytic cycle for the conversion of stored

TABLE 5. Retention of *Klebsiella pneumoniae* in lungs of mice and hamsters

NO ₂	No. of organisms/g of lung tissue			
	Control hamsters	Exptl hamsters	Control mice	Exptl mice
ppm				
5	3,948	2,966	3,366	2,457
25	—	—	1,530	1,092
35	7,284	4,253	—	—
50	7,142	3,122	1,289	883

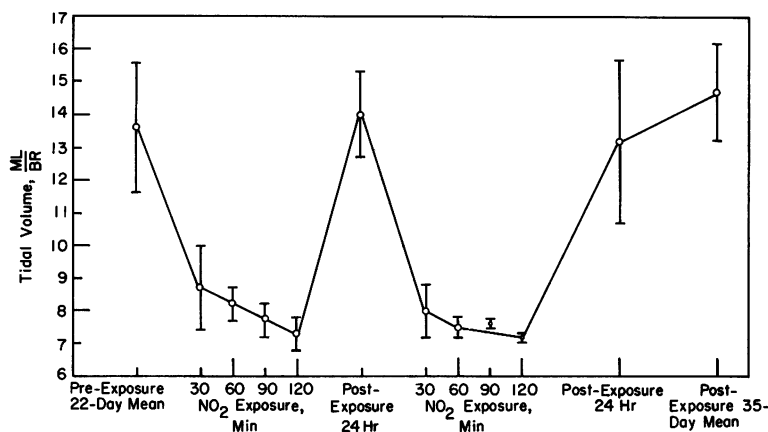


FIG. 4. Mean tidal volumes of monkey exposed to 35 ppm of NO₂.

energy. Recently, the enzyme has been separated electrophoretically into five components defined as LDH isoenzymes (29). Diseases, such as cardiac infarction (30), hepatitis (25), and cancer (23), produce abnormal serum and tissue isoenzyme patterns that are indicative of the tissues affected.

Exploratory studies were conducted to determine whether exposure to nitrogen dioxide and infection with *K. pneumoniae* produce an atypical LDH isoenzyme pattern in serum or selected tissues and whether the pattern is indicative of the resulting pathology. The limited number of animals (four) used per point did not permit an exhaustive analysis of the data. However, the differences obtained are large enough to suggest trends and to form the base for additional experimentation. Hamsters were exposed to 5 and 35 ppm of nitrogen dioxide for 2 hr and examined frequently over a 72-hr period.

Heart tissue was removed from each hamster; the LDH enzyme was extracted and resolved into isoenzyme components. Approximately 1 hr after exposure, in either group the enzymatic activity of isoenzymes 1 and 2 was reduced and remained depressed for approximately 1 day. Correspondingly, the isoenzyme activity in bands 4 and 5 increased. This period of altered isoenzyme activity coincides with the period of maximal susceptibility to respiratory infection after exposure to nitrogen dioxide. Hamsters subjected to infectious challenge only did not experience these alterations.

The livers were removed and the LDH isoenzyme was extracted. Although there was a large variability among the hamsters in a group, the overall trend was clear. Hamsters exposed to 5 or 35 ppm showed a decrease of approximately 50% in band 1 and 2 isoenzyme activity 5 hr after exposure.

Exploratory studies were also conducted with squirrel monkeys. One monkey was exposed to 35 ppm for 2 hr and subsequently was infected intravenously with *K. pneumoniae*. The LDH activity in the lung tissue increased 5-fold 24 hr after exposure. The increase in activity was accompanied by a marked and disproportionate increase in the LDH activity in bands 4 and 5.

Two monkeys exposed to 50 ppm of nitrogen dioxide and to *K. pneumoniae* aerosol displayed similar isoenzyme alterations. Upon autopsy, the lung tissue appeared gray, with patches of marked reddish congestion clearly demarcated from the gray areas. Tissue sections from both gray and red areas showed much interstitial and intralveolar edema, with congestion and cellular infiltration. Tissue excised from each of these areas produced abnormal isoenzyme patterns, each very different from the other. The red area produced one major isoenzyme band, band 5, with considerably reduced activity in the remaining isoenzyme fractions.

Current theories suggest that the LDH molecule is a tetrameric peptide molecule and that the synthesis of the molecule is controlled by two genes (5). One gene is responsible for the synthesis of LDH isoenzyme 1 and one for the synthesis of LDH isoenzyme 5. The three remaining isoenzymes are merely combinations of isoenzymes 1 and 5. Cahn, Kaplan, and associates (5) suggest that LDH isoenzyme 1 is associated with cells undergoing aerobic metabolism and LDH isoenzyme 5 with cells functioning anaerobically. Brody and Engel (4) have demonstrated that LDH activity is associated with the mitochondrial membrane and is readily dissociated when the tissue is manipulated during fixation. Therefore, rupture of the cellular membrane (cell death) would be indicated by an increase in serum LDH activity.

Altered metabolism induced by stress may be related to increased enzyme activity and altered isoenzyme ratios. Recently, Vesell (27) has published contradictory results. Studies conducted with nucleated red blood cells and normal red blood cells devoid of a nucleus indicated that LDH isoenzyme band 5 is located in the nucleus, and isoenzymes 1, 2, and 3 are located in the cytoplasm. Vesell therefore takes exception to the theories regarding the relationship of aerobic-anaerobic metabolism to isoenzymes 1 and 5.

The preliminary data acquired to date do not permit interpretation of the cellular mechanisms involved. Nevertheless, it is apparent that alteration of the isoenzyme ratios is related to pathology, and may ultimately provide information regarding altered cellular metabolism induced by the nitrogen dioxide and infectious challenge stress.

EFFECT OF CHRONIC EXPOSURE

Exposure to low levels of pollutants over extended periods of time are a threat to heavily populated communities. Air pollution surveys indicate a maximal concentration of 3.5 ppm of nitrogen dioxide. Daily variations in the concentration of nitrogen dioxide in a polluted atmosphere result from varying emission rates, wind velocity and direction, height of inversion layer, etc. The average 8-hr levels of oxides of nitrogen in one urban area on days with significant air pollution ranged from 0.1 to 0.5 ppm (26).

Several investigators have reported on the effect of chronic and intermittent exposures to nitrogen dioxide. Ronzani (24) concluded that repeated daily exposures to 100 ppm had no distinct acute effect in animals. Gray et al. (12) exposed rats to 9 to 14 ppm for 4 hr per day, 5 days per week, for 6 weeks. They observed an inflammatory condition spread throughout the entire respiratory tract. The same authors (13) found no evidence of pathology in rats, guinea pigs, and mice exposed daily for 6 months to 4 ppm.

Wagner et al. (28) exposed dogs, guinea pigs, rabbits, rats, hamsters, and mice to 1, 5, and 25 ppm for periods up to 18 months. At no exposure level did changes in body weight, hematological value, or biochemical index vary significantly from the control data. The respiratory functions in exposed rabbits were equivalent to those in the controls, with the exception of the 25-ppm group, which indicated a slight and transitory elevation in mean oxygen consumption. Detailed histological examination of tissues of animals sacrificed at various time intervals presented no evidence that nitrogen dioxide had any morphological effect. Their studies with a strain of mice

TABLE 6. Effect of continuous exposure to 0.5 ppm of nitrogen dioxide on mortality of mice challenged with *Klebsiella pneumoniae*

NO ₂ exposure	Mortality (deaths/total)			P
	Infected controls	Exptl group	Change	
			%	
7 days	187/280	189/280	1.0	
14 days	81/180	92/180	13.6	
1 month	26/60	34/60	30.9	
2 months	68/100	78/100	14.7	
3 months	64/100	92/100	43.7	<0.05
6 months	24/50	44/50	83.3	<0.001
9 months	38/70	49/70	28.9	<0.001

susceptible to spontaneous pulmonary tumor suggested a possible tumorigenic accelerating capacity of nitrogen dioxide.

In our studies, Swiss albino mice were exposed continuously, 24 hr per day, to 0.5 ppm of nitrogen dioxide. Three times a week, the mice were removed from the chamber for approximately 1 hr for maintenance and feeding. After various periods of nitrogen dioxide exposure, the mice were challenged with the aerosol of *K. pneumoniae* and maintained in a clean air atmosphere for 14 days after the challenge. Control animals were of the same age as the experimental mice, and were treated identically with the exception of the nitrogen dioxide exposure. The data in Table 6 show an increase in susceptibility to infection after 3 months of exposure to the gas. Some degree of linearity was observed when the arc transformed differences in mortalities were plotted against the duration of exposure to nitrogen dioxide (Fig. 5).

The effect of continuous and intermittent exposure to 0.5 ppm of nitrogen dioxide over a 30-day period was investigated. After 30 days of continuous exposure, Swiss albino mice were challenged by the respiratory route with airborne *K. pneumoniae*. The deaths were recorded during the next 14-day holding period at ambient atmosphere. For intermittent exposure, mice were exposed to 0.5 ppm for 6 hr a day, 5 days a week, for a total of 30 days before the infectious challenge. The data summarized in Table 7 show the significant mortality increase due to the intermittent exposure.

Exposure of Swiss albino mice to 1.5 ppm of nitrogen dioxide for periods ranging from 2 hr to 3 months prior to the infectious challenge resulted in mortality shown in Table 8. The increase in mortality was significant after exposures of 8 hr or longer. A corresponding reduction in

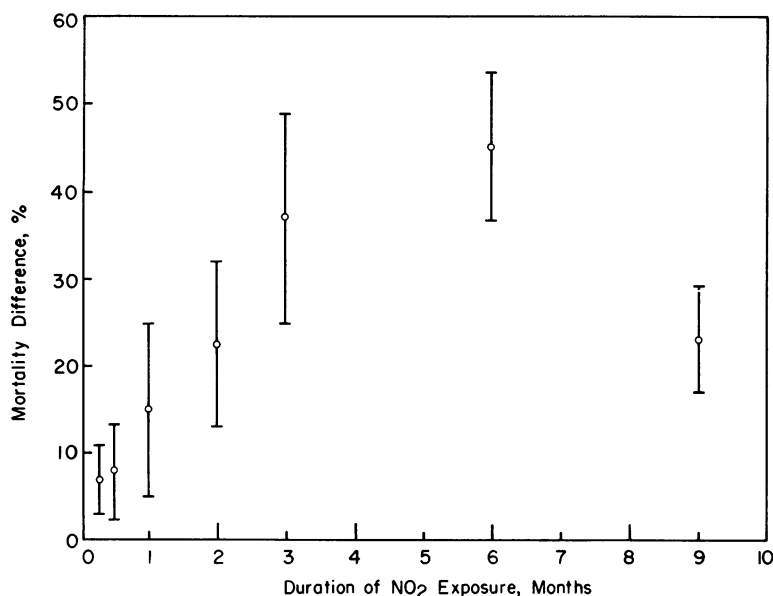
FIG. 5. Mortality difference versus chronic exposure to NO₂.

TABLE 7. Effect of continuous and intermittent exposure to 0.5 ppm of nitrogen dioxide for 30 days on mortality of infected mice

NO ₂ exposure	Mortality		Change	Survival time
	Deaths/total	Per cent		
Continuous			%	days
Controls.....	32/80	40.0		10.8
Experimental group.....	79/140	56.4	41.0	10.2
Intermittent				
Controls.....	22/60	36.6		12.2
Experimental group.....	57/80	71.3	94.8 ^a	9.2

^a Significant at *P* < 0.05.

the survival time occurred in all groups except the one exposed to nitrogen dioxide for 2 hr.

Exposure of infected mice to 1.5 ppm of nitrogen dioxide after the infectious challenge also increased mortality. The mortality in the control group challenged with the infectious agent only was 45%. After exposure to nitrogen dioxide for 2, 8, or 24 hr, the mortality rates were 80.0, 88.3 and 73.3%, respectively. The respective increases in mortality were 77.8, 96.2, and 62.9%, all three values being significant.

The significance of pre-exposure to nitrogen dioxide is further illustrated in Fig. 6. Three groups of mice were used. The one serving as the

TABLE 8. Effect of continuous exposure to 1.5 ppm of nitrogen dioxide on mortality of mice challenged with *Klebsiella pneumoniae*

NO ₂ exposure	Mortality (deaths/total)		Change
	Infected controls	Exptl group	
2 hr	45/90	51/90	13.4
8 hr	45/90	67/90	48.8 ^a
24 hr	45/90	59/90	31.2 ^a
7 days	20/40	28/40	40.0 ^a
14 days	17/40	39/40	129.4 ^a
90 days	23/50	70/100	52.3 ^a

^a Significant at *P* < 0.05.

control was challenged with *K. pneumoniae* aerosol and maintained in clean air after the infection. The second was infected and placed immediately after the challenge in an atmosphere of 1.5 ppm of nitrogen dioxide. The third was exposed to nitrogen dioxide for 24 hr, challenged, and returned to the 1.5-ppm atmosphere. Although the mortality of both groups exposed to nitrogen dioxide was higher than that of the control group, mice exposed to the gas both before and after the infectious challenge died faster, and ultimately the mortality in this group was the highest. The mortality at the end of the 30-day holding period was 58.3% for controls, 78.3% for mice exposed to nitrogen dioxide after the in-

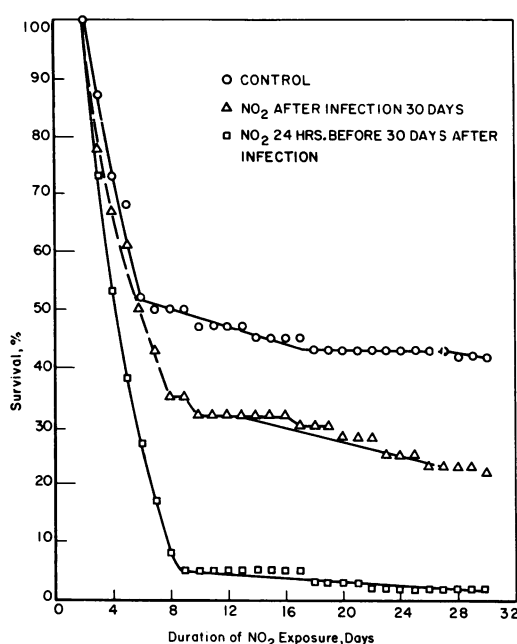


FIG. 6. Effect of pre-exposure of mice to nitrogen dioxide on resistance to *Klebsiella pneumoniae*.

fetious challenge, and 98.3% for mice exposed to nitrogen dioxide both before and after the infectious challenge.

EFFECT OF AGE

The effect of age on resistance to *K. pneumoniae* infection was investigated. Young mice 6 to 8 weeks old and weighing 20 ± 2 g, mice 6 months old and weighing 32 ± 2 g, and mice 9 months old and weighing 39 ± 2 g were maintained at ambient atmosphere and were challenged simultaneously with the infectious agent. Deaths were recorded for 14 days after the challenge. No significant differences were observed in the mortality rates among these groups. The mortality of the 6-month-old mice was 48.0; the 9-month-old mice, 54.2%; and the 6 to 8-week-old mice, 52.5%.

DISCUSSION AND CONCLUSIONS

The effects of exposure to nitrogen dioxide on man and on animals are confined almost exclusively to the respiratory tract. With increasing dosage, the progressive effects of this gas are: odor perception, nasal irritation, difficulty in breathing, acute respiratory irritation, edema, and death. Experimental and epidemiological data pertaining to nitrogen dioxide effects in man are sparse, especially in the low concentration level found in community air pollution.

In most species of laboratory animals, concentrations of nitrogen dioxide above 200 ppm produce death even after a single 5- to 15-min exposure. Continuous 30- to 60-min exposures to 100 to 200 ppm or 8-hr exposures to 50 ppm also produce death. Intermittent exposures of less than 50 ppm, on the other hand, are not fatal. Thus, it appears that the existence of a recovery period reduces mortality.

Lower concentrations, 10 to 20 ppm, produce pathological changes in the lungs. Continuous exposures to 5 or 10 ppm result in changes in the bronchial epithelium; lower concentrations produce only minor changes. Freeman and Haydon (10) observed minor changes in the bronchial epithelium after continuous exposure to 4 ppm for 20 weeks. Balchum et al. (1) showed that exposure of guinea pigs to 5 ppm produced minor pulmonary changes and demonstrated the development of circulating substances capable of agglutinating normal lung proteins.

The work reported in this paper suggests a more sensitive indicator of biological effects of nitrogen dioxide, namely, a synergistic effect or secondary effect, demonstrated by reduction in resistance to infection. A single 2-hr exposure of Swiss albino Webster strain mice or of inbred mice to 3.5 ppm of nitrogen dioxide before or after respiratory challenge with aerosol of *K. pneumoniae* significantly increased mortality. To produce the same effect in hamsters and squirrel monkeys, 35 ppm was required during the 2-hr exposure period. The effect of the single 2-hr exposure was not persistent, and a return to normal resistance to the infection was observed within 24 hr after the exposure to nitrogen dioxide.

Continuous exposures to 0.5 ppm for 3 months or longer as well as intermittent daily exposures over a 30-day period produced the same effect in mice.

Exploratory studies conducted to define the mechanisms responsible for the increased susceptibility to infection suggest that exposure to nitrogen dioxide permits better colonization of bacteria in the lungs of mice and hamsters.

Exposure to 25 to 35 ppm of nitrogen dioxide affected the pulmonary function in squirrel monkeys. Similar observations in guinea pigs were reported by Murphy et al. (19). The respiratory rate increased and the tidal volume decreased in guinea pigs exposed to 5.2 and 13.0 ppm of nitrogen dioxide. The time of onset of the respiratory changes was inversely related to the concentration of the inhaled gas. When the guinea pigs were returned to clean air, the pulmonary function gradually returned to the pre-exposure level.

Extrapolation of the effects of nitrogen dioxide on resistance to *K. pneumoniae* infection of man, or that due to other species of pathogenic microorganisms, can be speculative only. However, the work is significant in pointing to possible relationships between air pollutants and changes in resistance to respiratory infection.

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